Pathogenicity and real-time PCR detection of *Fusarium* spp. in wheat and barley roots

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Abstract: The pathogenicity of five *Fusarium* spp. frequently isolated from wheat and barley roots in southern Idaho was investigated during four growth-chamber experiments and two field studies. A real-time PCR assay for quantifying the presence of *F. culmorum* from infected root tissue was also developed based on nucleotide sequence for the *tri5* gene. *Fusarium culmorum*, followed by *F. acuminatum* and *F. reticulatum*, resulted in the largest root lesions and percent infected root area. However, *F. semitectum*, followed by *F. acuminatum* and *F. equiseti*, had the greatest impact on total root length. The TaqMan-based real-time assay was able to quantify *F. culmorum* in root tissue from both growth-chamber and field studies down to 61 pg. The assay also detected *F. pseudograminearum* and *F. graminearum* but could not distinguish among these three *Fusarium* spp.

Key words: *Fusarium* spp., fusarium root rot, quantitative detection, trichothecene, wheat, barley.

Résumé : Le pouvoir pathogène de cinq espèces de *Fusarium* fréquemment isolées de racines de blé et d'orge dans le sud de l'Idaho a été étudié lors de quatre expériences en chambre de croissance et de deux autres en champ. Un test de réaction en chaîne de la polymérase en temps réel, basé sur la séquence de nucléotides du gène *tri5*, a aussi été développé pour quantifier la présence du *F. culmorum* provenant de tissu racinaire infecté. Le *F. culmorum*, suivi du *F. acuminatum* et du *F. reticulatum*, causait les lésions racinaires les plus larges ainsi que le pourcentage de surface racinaire infectée le plus important. Cependant, le *F. semitectum*, suivi du *F. acuminatum* et du *F. equiseti*, avait l'impact le plus grand sur la longueur totale des racines. Un test en temps réel TaqMan a permis de quantifier aussi peu que 61 pg de *F. culmorum* dans le tissu racinaire, tant dans les études en chambre de croissance que dans celles au champ. Le test peut aussi détecter le *F. pseudograminearum* et le *F. graminearum*, mais il ne peut distinguer les trois espèces de *Fusarium*.

Mots clés : *Fusarium*, piétin fusarien, détection quantitative, trichothécène, blé, orge.

Introduction

Fusarium root and foot rot of wheat is a concern worldwide (Cook 1981). The primary *Fusarium* causal agents of root rot are *F. culmorum* (Wm.G. Sm.) Sacc. and *F. pseudograminearum* Aoki & O'Donnell (Cook 1981; Strausbaugh et al. 2004; Wallwork et al. 2004). In some areas, *F. avenaceum* (Fr.) Sacc., *F. acuminatum* Ell. & Ev., *F. tricinctum* (Corda) Sacc., *F. equiseti* (Corda) Sacc., *F. reticulatum* Mont., *F. poae* (Peck) Wollenw., and *F. crookwellense* Burg. et al. have also been associated with the root-rot complex along with *Bipolaris sorokiniana* (Sacc.) Shoemaker (Cook 1980, 1981; Hill et al. 1983, 1987; Kane et al. 1987; Specht and Rush 1988; Strausbaugh et al. 2004; Wiese 1987). In southeastern Idaho (Intermountain West area of the United States), *F. culmorum* is the primary cause of fusarium root rot. Traditionally, soilborne pathogens have been managed through crop rotations, proper fertilization, tillage, and host resistance. However, crop rotation options are limited in some areas because of low rainfall. Also, in an effort to minimize production inputs and reduce soil and wind erosion, there is a trend toward direct-seeded cereal production (Strausbaugh et al. 2004). These cropping challenges along with the lack of commercial cultivars with resistance to fusarium root and foot rot makes controlling this disease complex problematic.

Aboveground symptoms of fusarium root and foot rot include missing and stunted plants and (or) the development of "white heads". However, these symptoms may not always appear, and fusarium root and foot rot may go unnoticed unless roots and crowns are examined. Root exam-

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ination is laborious and difficult, since lesions caused by *F. culmorum* and *F. pseudograminearum* can be confused with those caused by other *Fusarium* spp. and *B. sorokiniana*. Therefore, a quantitative molecular assay for the primary pathogens in the fusarium root rot complex would be beneficial.

The first molecular markers for *Fusarium* spp. were end-point assays limited to detection and identification (Chelkowski et al. 1999; Doohan et al. 1998; Nicholson et al. 2004; Niessen and Vogel 1998; Schilling et al. 1996; Turner et al. 1998; Williams et al. 2002). Next, competitive PCR (Nicholson et al. 1998) and SYBR-green real-time assays (Schnerr et al. 2001) were developed. However, end-point assays suffer from laborious end-point manipulations such as gel electrophoresis; the competitive assays have some degree of quantification, but suffer from putative exhaustion of reaction components, and since SYBR green binds indiscriminately to double-stranded DNA, these assays must be optimized carefully to avoid false positives (Bluhm et al. 2004; Waalwijk et al. 2004). Recently, molecular assays based on sequence-specific probes labeled with a fluorescent reporter and quencher, and combined with flanking primers, identified and quantified specific fungi in plant tissues. These real-time quantitative PCR assays give higher levels of specificity and sensitivity, shorter run times, and simpler and more rapid data analysis (Bluhm et al. 2004; Waalwijk et al. 2004).

Fusarium culmorum, along with some other *Fusarium* spp., produce zearalenone and a range of trichothecenes including deoxynivalenol, 15-acetyl-deoxynivalenol, nivalenol, and 4-acetyl-deoxynivalenol (Tóth et al. 2004). Although not necessary for initial infection, trichothecenes have been shown to be involved in pathogenesis (Tóth et al. 2004). The *tri5* gene, encoding trichodiene synthase, which is the first and committing step in the synthesis of trichothecene mycotoxins, has been suggested as a good target for a quantitative molecular assay (Waalwijk et al. 2004).

The development of germ plasm with improved resistance to fusarium root rot will be facilitated by combining a quantitative molecular approach targeted at the *tri5* gene with root-disease assays. *Fusarium culmorum* was previously identified as the primary causal agent for fusarium root rot in southeastern Idaho (Strausbaugh et al. 2004), but other *Fusarium* spp. were not investigated for their influence on root length. Thus the objective of this research was: (1) to investigate the ability of five *Fusarium* spp. to infect and influence roots and (2) to establish a real-time PCR assay based on the *tri5* gene and TaqMan technology for the causal agents *F. culmorum* and *F. pseudograminearum*.

Materials and methods

Reference cultures

Reference cultures were obtained from the Fusarium Research Center, University Park, Pennsylvania, United States, for the following *Fusarium* spp.: *F. culmorum* (R-9423), *F. pseudograminearum* (R-6563), *F. graminearum* Schwabe (R-7005), and *F. avenaceum* (R-6554). Representative cultures of the other *Fusarium* spp. (*F. culmorum* F70, F82, F83, F99; *F. acuminatum* F24, F59, F63, F146, F92; *F. equiseti* F55, F61, F67, F98; *F. semitectum* Berk. & Rav. F159; and *F. reticulatum* F60) utilized in this research were submitted to the Fusarium Research Center for identification as part of a previous research project (Strausbaugh et al. 2004). These representative cultures were used for comparison to identify the 10 *Fusarium* isolates utilized in the growth-chamber studies based on techniques and descriptions established by Nelson et al. (1983).

Growth-chamber studies

Ten *Fusarium* isolates (*F. culmorum* F546 and F422, *F. reticulatum* F421 and F404, *F. acuminatum* F425 and F396, *F. equiseti* F369 and F374, *F. semitectum* F376 and F378), taken from roots in the summer of 2003, were screened through experiments in growth chambers at low- and high-temperature ramping settings during the winter of 2003 and spring of 2004. Culture F546 originated from spring malting barley 'Harrington' in Bonneville County, Idaho, while the other nine cultures were isolated from winter wheat 'Eltan' in Power County, Idaho. A reference culture of *F. culmorum* (R-9423) and an uninoculated control were included in all experiments. Experiments were repeated once. Inocula of *Fusarium* isolates were produced by growing single-spore or hyphal-tip cultures on Difco® potato dextrose agar (PDA; Becton, Dickinson & Co., Sparks, Md.) in petri dishes. From these plates, five 5-mm plugs were placed in 125-mL flasks containing a sand–cornmeal mixture (95 g of 70-grit sand, 5 g of cornmeal, and 20 mL of water) that had been sterilized (40 min at 121 °C) in an autoclave. The inoculum flasks were placed on a laboratory bench at 21 °C for 1 week and shaken daily to redistribute the fungus. The isolates were screened in a growth chamber (Enconair® model GC-16) in 473-mL plastic drinking cups with holes in the bottom for water drainage. The cups were filled with dry vermiculite and slightly compressed to a 8-cm depth. Approximately 30 g of sand–cornmeal-based inoculum was layered onto the vermiculite and covered with 1.5 cm of vermiculite. Four seeds of the soft white winter wheat 'Brundage' or malting barley 'Harrington' were disinfected with 0.5% NaOCl for 3 min, rinsed in sterile water, air-dried in a laminar flow hood, and then placed on top of the vermiculite. The seeds were covered with 1.5 cm of vermiculite and compressed gently. The cups were watered and arranged on the growth-chamber bench in a randomized complete block design with four replications. We used the temperature-ramp method in the growth chamber, with a 14-h photoperiod (07:00–21:00) and a light intensity of 600–800 μmol m^{-2} s^{-1}. The settings for the low-temperature experiments were 10 °C from 24:00 till 08:00, followed by 14 °C at 08:00, then 18 °C at 09:00, and 22 °C from 10:00 till 22:00. Then, temperature reduction began: 18 °C at 22:00, followed by 14 °C from 23:00 till 24:00. For the high-temperature experiments, all temperatures were raised 6 °C above those for the low-temperature experiments, using the same temperature ramping procedure. After 10 days, the plants were removed from the cups, and the vermiculite was rinsed off the roots. Two plants in each cup were arbitrarily chosen for rating. On the two plants, both the main root and lesion length were measured with a ruler. Average root length, lesion length, and percent infection ([lesion length/root length] × 100) were calculated. The total root biomass for each plant was blotted dry, placed in

a plastic bag, and stored at –80 °C until DNA was extracted.

Field studies

Field studies were established in Bonneville and Power counties, in commercial dryland fields with 15-cm standing stubble and only natural inoculum present. The fields had been direct seeded to wheat and barley in preceding years with a one-pass direct-seed drill. The soil type in Bonneville County was a Tetonia silt loam classified as a coarse-silty, mixed, calcic, pachic cryoboroll with a pH of 6.3 and 2.6% organic matter. The soil type in Power County was a Newdale silt loam classified as a coarse-silty, mixed, frigid, calciorthidic haploxeroll with a pH of 7.6 and 1% organic matter. Experimental units were (3.66 m × 14.63 m) arranged in a randomized complete block design with four replicates. Treatments were an untreated control and methyl bromide fumigation. The fumigation was performed by placing methyl bromide under a plastic tarp at a rate of 45 g/m^2 on 24 September 2002. The tarp was removed after 7 days. Prior to planting, all seed was treated with Gaucho® 480 (1.95 mL/kg of seed) for insect control.

Seed of the spring malting barley 'Harrington' was planted at 89.6 kg/ha in Bonneville County on 21 April 2003 with a direct-seed drill with Conserva Pak® openers. The openers placed the fertilizer (72.79 kg N, 28.0 kg P_2O_5, and 17.93 kg S per hectare) 3.81 cm below the seed in a one-pass operation. The weeds and volunteer wheat were controlled by a glyphosate spray at 1.17 L/ha on 24 April 2003. On 26 June 2003, 20 plants were dug and rated for root diseases.

Seed of the winter wheat 'Eltan' was planted at 112 kg/ha in Power County on 15 October 2002 with a direct-seed drill with Conserva Pak openers. The openers placed the fertilizer (16.79 kg N and 11.21 kg P_2O_5 per hectare) 3.81 cm below the seed in a one-pass operation. The weeds and volunteer wheat were controlled by a glyphosate spray 1.17 L/ha on 11 October 2002. On 13 June 2003, 20 plants per experimental unit were dug and rated for root diseases.

The plants rated for root diseases were dug at Feekes growth stage 10.1 to 10.5 (late boot to flowering). Main stem and seminal roots were evaluated for disease severity and assigned to one of the following root-lesion categories: spear tips, discrete-black lesions, and diffuse-brown lesions. The spear-tip lesion type included roots with spear tips and lesions associated with reduced root diameter. These lesions are usually associated with *Rhizoctonia* spp., but the pathogen was difficult to isolate from mature root systems. The diffuse-brown lesion type included roots with diffuse-brown to dark-brown lesions, but no reduction in root diameter. Isolations from this lesion type were *Fusarium* spp. and *B. sorokiniana*. The discrete-black lesion type had no reduction in root diameter and is normally associated with *Gaeumannomyces graminis* (Sacc.) Arx & D. Oliver var. *tritici* J. Walker. When isolated, *Gaeumannomyces graminis* var. *tritici* was always associated with this lesion type, but *Fusarium* spp. and *B. sorokiniana* were occasionally isolated. Severity was based, on a 0 to 4 scale, on the percentage of roots with lesions: 0, no lesions; 1, <10%; 2, 10%–33%; 3, 34%–66%; and 4, >66%. A disease severity index (DSI) was calculated for each plot by (% incidence × mean severity)/4. Isolations were made from root lesions (four lesions per type per plot in each field) after surface disinfesting roots in a 0.5% NaOCl solution for 30 s, splitting the root lesions in half, and placing one half of the lesions on 1.8% Bacto® agar (Becton, Dickinson & Co.) and the other half on Difco PDA. Both media were amended with streptomycin sulfate (200 mg/L). Fungi growing from the roots were transferred to a plate of streptomycin-amended PDA. All fungal isolates were then single spored (or hyphal tipped if no spores were present) on Bacto agar, and then transferred to PDA. Fungal identifications were performed under a light microscrope. The *Fusarium* isolates were also grown on synthetic, nutrient-poor agar (Gerlach and Niremberg 1982) with pieces of sterile (autoclaved for 15 min at 121 °C) filter paper and carnation leaves placed on the agar. The *Fusarium* cultures were placed under fluorescent light for 3 weeks and identified as described by Nelson et al. (1983). Main stem and seminal roots from half the rated plants were saved in plastic bags and held at –80 °C for DNA extraction.

DNA extraction

The fungal isolates were grown in potato dextrose broth in 125-mL plastic culture flasks at 21 °C while shaken at 125 r/min for 5 days. The fungal growth and broth were strained through cheesecloth, rinsed with reverse-osmosis water, placed in a 1.5-mL Eppendorf tube, and frozen at –80 °C in a freezer. DNA extractions were performed following a "mini-prep" method developed by Afanador et al. (1993) and modified by Strausbaugh et al. (2003).

PCR primer and probe design

Nucleotide sequences corresponding to gene *tri5* encoding trichodiene synthase from *Fusarium* spp. were obtained from GenBank (*F. poae*, accession No. U15658; *Gibberella zeae* (Schwein.) Petch, accession No. U22464). A sequence alignment was performed, based on the most highly conserved region (bases 800–1700) (Niessen and Vogel 1998) of *tri5* gene, to design the primer and probe set. Within the *Gibberella zeae* sequence (accession No. U22464) the primers were designed to amplify the region 866 through 928 and the probe sits at 887–910. The primers and probe were designed using Primer Express software (Applied Biosystems, Foster City, Calif.).

PCR amplification

The real-time PCR reactions were performed oil-free in volumes of 25 µL that contained: 12.5 µL of TaqMan Universal PCR Master Mix (Applied Biosystems), 5 µL of DNA template (= 75 ng of total DNA), 2.5 µL (3 µmol/L) of Gzeae87T forward primer (5′-GCGCATCGAGAATTTGCA-3′), 2.5 µL (3 µmol/L) of Gzeae87T reverse primer (5′-TGGCGAGGCTGAGCAAAG-3′), and 2.5 µL (2 µmol/L) of Gzeae87T probe (5′-6FAM-TGCTTACAACAAGGCTGCCCACCA-TAMRA-3′). Amplifications were performed using an ABI PRISM 7900HT (Applied Biosystems) set to emulate an ABI PRISM 9600 cycler. The instrument was programmed for 2 min at 50 °C, 5 min at 95 °C, and then 37 cycles of 20 s at 95 °C and 1 min at 60 °C. To avoid artifacts from primer dimers, a cut-off value of 36 was established for C_t, the cycle number at which the fluorescence

generated within the reaction crosses the threshold for being significantly different from the baseline or background signal. The calibration curve was produced with DNA extracted from the *F. culmorum* (R-9423) reference culture. The DNA concentrations in the curve included 10 increases from 0.145 to 75 ng in twofold steps. After the first two studies, five increases in tenfold steps covered the region from 0.0061 to 61 ng. To confirm that seed-borne contaminated roots contained *F. culmorum* DNA, we ran assays with the *F. culmorum* sequence-characterized amplified region (SCAR) marker Fc01 and *F. graminearum* SCAR marker Fg16N following protocols established by Nicholson et al. (1998).

Statistical analyses for all studies were performed with SAS version 8.2 (SAS Institute Inc. 1999). Data were analyzed according to the general linear model procedure (SAS Institute Inc. 1999). Mean comparisons were performed using Fisher's protected least significant difference (LSD). Homogeneity of variances was determined with Bartlett's test.

Results

Growth-chamber studies

Data from replications run at the same temperature and on the same host for three of the growth-chamber studies (Tables 1–3) were not combined for analysis, since root length was significantly different (P range, 0.02–0.0013); this also affected the calculation for percent root area infected. However, since repeated studies had the same trend, we present only one set of data from each set of studies to avoid redundancy. Data from both replications of the high-temperature barley experiments (Table 4) were combined, since they did not differ significantly (P range for parameters, 0.109–0.477) and variances were homogeneous. In all four growth-chamber studies, *F. culmorum* always resulted in significant root lesions and percent root area infected, but total root length did not always differ from that of the control. Based on the percent root area infected, the average ranking across all experiments for *F. culmorum*, *F. acuminatum*, *F. reticulatum*, *F. semitectum*, and *F. equiseti* was 2.4 (most area infected), 4.7, 6.3, 7.6, and 9.6 (least area infected), respectively. Based on lesion size, the average ranking across all experiments for *F. culmorum*, *F. reticulatum*, *F. acuminatum*, *F. semitectum*, and *F. equiseti* was 2.0 (largest lesions), 5.4, 5.5, 9.3, and 9.4 (smallest lesions), respectively. Based on total root length, the average ranking across all experiments for *F. semitectum*, *F. acuminatum*, *F. equiseti*, *F. reticulatum*, and *F. culmorum* was 9.2 (least root length), 8.7, 7.6, 5.8, and 4.5 (most root length), respectively. Thus, these data indicate that *F. culmorum* produces the largest root lesions but has very little influence on root length. The opposite could be said for *F. semitectum*, *F. acuminatum*, and *F. equiseti*, since they produce the smallest root lesions but have the greatest impact on root length.

The high-temperature wheat studies had longer roots (50.9 cm vs. 38.1 cm, $P < 0.0001$) and larger lesions (4.5 cm vs. 3.1 cm, $P = 0.0011$), but there was no difference in the percent root infected (9.5% vs. 9.4%, $P = 0.4575$) in comparison with the low-temperature studies. The high-temperature barley studies had longer roots (69.7 cm vs. 61.0 cm, $P < 0.0129$) but similar lesions (6.9 cm vs. 6.6 cm, $P = 0.3493$) and percent root infected (13.1% vs. 11.7%, $P = 0.2421$) as the low-temperature studies. In a crop comparison, the barley had longer roots (65.3 cm vs. 44.8 cm, $P < 0.0001$), larger lesions (6.8 cm vs. 3.8 cm, $P < 0.0001$), and more percent root infected (12.4% vs. 9.5%, $P = 0.0034$) when compared with the wheat.

PCR amplification

Pure cultures of *F. culmorum* (R-9423), *F. pseudograminearum* (R-6563), and *F. graminearum* (R-7005) were detectable in the real-time PCR assay based on the *tri5* gene. The assay was also successful in detecting *F. culmorum* in both wheat and barley roots in growth-chamber and field assays (Tables 1–5). The assay did not not amplify the sequence of *tri5* gene from pure cultures of *F. avenaceum* (R-9576, R-6554), *F. acuminatum* (F193, F195, F333), *F. reticulatum* (F421), *F. semitectum* (F242, F244, F250, F251, F321), *F. equiseti* (F208, F339), *Rhizoctonia solani* (104-G-B, 132-G-B, 157-F-A, 157-R-C), *Gaeumannomyces graminis* var. *tritici* (151-R-C, 162-G-A), *Pythium* spp., and the 10 most common fungal saprophytes isolated from wheat and barley roots in southeastern Idaho. The amount of detectable DNA for the *F. culmorum* isolates were always higher in the barley growth-chamber assays than the wheat assays at the same temperature, indicating that there was more fungal development on the barley. The lesion size on barley was always larger as well, which aids in correlating real-time assay results.

When generating the calibration curve with *F. culmorum* DNA, we could conservatively detect down to 61 pg (C_t = 35–36), using 37 cycles when annealing and extending at 60 °C. The r^2 values for all real-time experiments ranged from 0.99 to 1.00 for the straight line generated when plotting the log of the DNA concentration versus the cycle number.

The real-time PCR assay always detected a strong response in roots inoculated with *F. culmorum*. Some occasional seed-borne contamination (discolored root tissue at seed attachment) was detected in roots inoculated with other *Fusarium* spp. and in uninoculated controls, but it was always well below (except in experiment 0411) the level detected in roots inoculated with *F. culmorum*. The seed-borne contamination was tested with the *F. culmorum* SCAR marker Fc01 (found to be positive) and the *F. graminearum* SCAR marker Fg16N (found to be negative). The C_t values always correlated well with the percent root area infected based on Spearman's rank correlation (Table 5).

Field studies

At the Ririe location (Bonneville County) in 2003, we recorded the following fungi, based on 245 root-lesion isolations: 67% *F. culmorum*, 20% *B. sorokiniana*, 7% *F. equiseti*, 4% *F. acuminatum*, 1% *F. reticulatum*, and 1% *F. semitectum*. Based on disease-severity ratings for the seminal and main roots, the response to methyl bromide fumigation (DSI = 26.8) was significant ($P = 0.0292$) compared with the nontreated control (DSI = 42.0). The real-time assay was able to quantify *F. culmorum* in bulked root samples from all untreated plots, while *F. culmorum* was

Table 1. Pathogenicity experiment (0407) conducted on soft white winter wheat 'Brundage', using *Fusarium* isolates, under low-temperature growth-chamber conditions.

Culture	*Fusarium* spp.	Total root length (cm)	Lesion length (cm)	Percent root infected	DNA (pg)[a]
R-9423	*F. culmorum*	32	5.8	22	1849
F546	*F. culmorum*	36	6.8	21	2243
F422	*F. culmorum*	33	6.5	20	3633
F396	*F. acuminatum*	31	2.6	10	—
F421	*F. reticulatum*	34	2.7	10	—
F404	*F. reticulatum*	41	3.9	10	—
F376	*F. semitectum*	25	2	8	—
F374	*F. equiseti*	23	1.6	8	—
F425	*F. acuminatum*	33	2.2	8	—
F369	*F. equiseti*	21	0.7	5	—
F378	*F. semitectum*	42	0.8	2	—
Control		48	0.2	0	—
P* >*F		0.0002	<0.0001	<0.0001	0.113
LSD		11	1.5	7	NS

Note: —, no data since there was no fluorescence detected; LSD, least significant difference for $P \leq 0.05$; NS, not significantly different.

[a]Detectable DNA concentration, based on real-time detection of *tri5* gene, associated with C_t, the cycle number at which the fluorescence generated within the reaction crosses the threshold for being significantly different from the baseline or background signal. Analyses were performed on the treatments where fluorescence was detected, in all experimental units. There was some detectable *F. culmorum* seed-borne contamination in roots from two control plants (1543 and 149 pg, respectively). These control plants had visible lesions next to seed attachment, which accounts for the small amount of percent infected root. Two other contaminated plants were detected: one F374- and one F376-inoculated plant, with 262 and 807 pg, respectively.

Table 2. Pathogenicity experiment (0411) conducted on soft white winter wheat 'Brundage', using *Fusarium* isolates, under high-temperature growth-chamber conditions.

Culture	*Fusarium* spp.	Total root length (cm)	Lesion length (cm)	Percent root infected	DNA (pg)[a]
F546	*F. culmorum*	58	14.6	25	10 464
R-9423	*F. culmorum*	65	14	22	9 216
F422	*F. culmorum*	54	9.9	20	6 184
F404	*F. reticulatum*	42	4.6	14	—
F425	*F. acuminatum*	30	4.1	14	—
F396	*F. acuminatum*	25	3.6	14	—
F376	*F. semitectum*	41	3.1	8	—
F421	*F. reticulatum*	55	2.4	8	—
F378	*F. semitectum*	39	1.7	6	—
F374	*F. equiseti*	28	1.4	4	—
F369	*F. equiseti*	44	1	4	—
Control		56	0.6	1	4 740
P* >*F		<0.0001	<0.0001	<0.0001	0.02
LSD		16	3.1	8	3 227

Note: —, no data since there was no fluorescence detected; LSD, least significant difference for $P \leq 0.05$.

[a]Detectable DNA concentration, based on real-time detection of *tri5* gene, associated with C_t, the cycle number at which the fluorescence generated within the reaction crosses the threshold for being significantly different from the baseline or background signal. Analyses were performed on the treatments where fluorescence was detected, in all experimental units. There was detectable *F. culmorum* seed-borne contamination next to seed attachment in the controls and, therefore, these were included in the analysis. Seed-borne contamination was also detected in one F376- and two F378-inoculated plants, with 531, 1033, and 126 pg, respectively.

detectable only in three out of eight fumigated plots. When comparing the ranks of the bulks based on DSI and C_t, using Spearman's rank correlation, no correlation was found ($r_s = 0.230$, $P = 0.3917$). Given that only 67% of the lesions were attributed to *F. culmorum*, it is not surprising that we might have trouble establishing a correlation.

At the Arbon Valley location (Power County) in 2003, we recorded the following fungi, based on 307 root-lesion isolations: 31% *F. reticulatum*, 20% *F. equiseti*, 18% *F. acuminatum*, 11% *F. semitectum*, 8% *Gaeumannomyces graminis* var. *tritici*, 3% *F. chlamydosporum* Wollenw. and Reinking, 1% *F. crookwellense*, 1% *F. culmorum*, 1%

Table 3. Pathogenicity experiment (0431) conducted on malting barley 'Harrington', using *Fusarium* isolates, under low-temperature growth-chamber conditions.

Culture	*Fusarium* spp.	Total root length (cm)	Lesion length (cm)	Percent root infected	DNA (pg)[a]
F422	*F. culmorum*	86	16.1	20	16 832
F396	*F. acuminatum*	40	5.3	20	—
F546	*F. culmorum*	96	15.6	17	7 884
R-9423	*F. culmorum*	87	12.8	17	12 271
F425	*F. acuminatum*	87	4.8	8	—
F376	*F. semitectum*	46	2.6	8	—
F404	*F. reticulatum*	61	4.1	8	—
F421	*F. reticulatum*	70	4.9	7	—
F369	*F. equiseti*	43	1.3	4	—
F374	*F. equiseti*	71	1.5	2	—
F378	*F. semitectum*	70	0.5	1	—
Control		116	0	0	—
P* >*F		<0.0001	<0.0001	0.0006	0.3139
LSD		28	3.9	10	NS

Note: —, no data since there was no fluorescence detected; LSD, least significant difference for $P \leq 0.05$; NS, not significantly different.

[a]Detectable DNA concentration, based on real-time detection of *tri5* gene, associated with C_t, the cycle number at which the fluorescence generated within the reaction crosses the threshold for being significantly different from the baseline or background signal. Analyses were performed on the treatments where fluorescence was detected, in all experimental units. There was detectable *F. culmorum* seed-borne contamination in roots from one control cup (138 pg).

Table 4. Pathogenicity experiments (0427 and 0428) conducted on malting barley 'Harrington', using *Fusarium* isolates, under high-temperature growth-chamber conditions.

Culture	*Fusarium* spp.	Total root length (cm)	Lesion length (cm)	Percent root infected	DNAa (pg)[a]
F546	*F. culmorum*	54	15.1	36	23 304
R-9423	*F. culmorum*	64	18.6	32	18 294
F422	*F. culmorum*	72	19.4	29	22 473
F425	*F. acuminatum*	42	6.1	17	—
F396	*F. acuminatum*	49	4.8	11	—
F421	*F. reticulatum*	72	4.9	8	—
F376	*F. semitectum*	52	2.9	6	—
F404	*F. reticulatum*	69	3.8	6	—
F369	*F. equiseti*	45	1.9	5	—
F374	*F. equiseti*	73	3.1	4	—
F378	*F. semitectum*	44	1.2	3	—
Control		94	1.1	1	718
P* >*F		<0.0001	<0.0001	<0.0001	<0.0001
LSD		12	3	7	1 538

Note: —, no data since there was no fluorescence detected; LSD, least significant difference for $P \leq 0.05$.

[a]Detectable DNA concentration, based on real-time detection of *tri5* gene, associated with C_t, the cycle number at which the fluorescence generated within the reaction crosses the threshold for being significantly different from the baseline or background signal. Analyses were performed on the treatments where fluorescence was detected, in all experimental units. In 0427, *F. culmorum* seed-borne contamination was detectable in one F378- and two F421-inoculated plants, with 310, 557, and 240 pg, respectively. In 0428, *F. culmorum* seed-borne contamination was also detectable in one F369- and two F376- and F378-inoculated plants, with 166, 636, 872, 286, and 585 pg, respectively.

Rhizoctonia spp., and 6% other *Fusarium* spp. Based on disease-severity ratings for the seminal and main roots, the response to methyl bromide fumigation (DSI = 4.0) was significant ($P = 0.0033$) compared with the nontreated control (DSI = 39.3). The real-time assay was not able to detect and quantify *Fusarium* spp. in bulked root samples. Given the low incidence of *F. culmorum* at this location, these results are encouraging. These data indicate that *Fusarium* spp. found in Idaho, other than *F. culmorum*, *F. pseudograminearum*, or *F. graminearum*, are unlikely to be detected with this molecular assay. These data also confirm that saprophytic fungi are not being detected.

Discussion

Fusarium culmorum caused the largest root lesions on both wheat and barley in growth-chamber assays and was precisely quantified utilizing a quantitative real-time PCR

Table 5. Correlation of the ranks for C_t values (ascending) and percentage of infected root (descending) for all plants with detectable *Fusarium culmorum* DNA ($C_t \leq 36$) from growth-chamber experiments.

Experiment	Spearman's rank correlation	Probability
406	0.732	0.0029
407	0.587	0.0274
410	0.749	0.002
411	0.718	0.0026
427	0.754	0.0012
428	0.903	<0.0001
431	0.736	0.0041
432	0.719	0.0038

molecular assay based on the *tri5* gene. *Fusarium acuminatum* and *F. reticulatum* also frequently caused significant root lesions. However, despite forming only small or even insignificant lesions (at least in appearance), *F. semitectum*, followed by *F. acuminatum* and *F. equiseti* caused the largest reductions in total root length.

In the Pacific Northwest, fusarium root rot has been attributed to *F. culmorum* and *F. pseudograminearum*, and to a lesser extent, to *F. avenaceum* (Cook 1980; Paulitz et al. 2002; Smiley and Patterson 1996; Smiley et al. 1996). In the adjacent Intermountain West (IMW), fusarium root rot has been attributed to *F. culmorum*, although some isolates of *F. equiseti*, *F. reticulatum*, and *F. acuminatum* were found to be pathogenic (Strausbaugh et al. 2004). In Saskatchewan, the primary *Fusarium* spp. other than *F. culmorum* isolated from wheat roots were *F. equiseti* and *F. acuminatum* (Bailey et al. 2001). This similarity in the fusarium root-disease complex between Saskatchewan and the IMW is likely related to the similar climatic conditions of the two regions. Other generally arid areas of the United States such as Wyoming, Colorado, and Texas have also reported *F. acuminatum* and *F. equiseti* to be associated with the fusarium root rot complex (Hill et al. 1983; Specht and Rush 1988). However, *F. acuminatum* and *F. equiseti* have generally been considered to be weak or minor pathogens (Specht and Rush 1988; Strausbaugh et al. 2004). Isolates of *F. equiseti* from Texas were found to be generally nonpathogic on 2-week-old seedlings (Specht and Rush 1988). Data presented in Tables 1–4, and previously published data from IMW (Strausbaugh et al. 2004), would support that *F. equiseti*, *F. acuminatum*, *F. reticulatum*, and *F. semitectum* are minor pathogens if only lesion size and the percent root area infected were analyzed. However, if these fungi significantly shorten root length despite the paucity of obvious root rot symptoms, they may be significant pathogens. The data presented in Tables 1–4 were generated in soilless assays under conditions that do not reflect moisture-limiting conditions. If these so called minor pathogens significantly restrict root growth in our drier, rain-fed cereal-production areas such as Power County, they are likely to impact production. In the rain-fed cereal-production areas of the IMW, it is not uncommon to have moisture-limiting conditions in the upper 3 feet (1 feet = 0.3048 m) of the soil profile when the heads are filling (C.A. Strausbaugh, unpublished data). Thus we would anticipate a potentially negative impact on production if root growth is restricted by *Fusarium* spp. that produce small, visually insignificant root lesions. These "minor pathogens" should be investigated for their ability to limit root growth in a soil system with competing soil microbes under moisture stress normally associated with rain-fed cereal production in arid areas. In the Pacific Northwest, *Fusarium* root and (or) crown infections were found to be more severe under drought conditions (Smiley et al. 1996), leading to discoloration of the foot of the plant. Moisture stress alone will influence the severity of *Fusarium* infections, but an excessive amount of available nitrogen in relation to the amount of available soil moisture can also lead to severe foot rot (Smiley et al. 1996). Thus, the interaction of available nitrogen, soil moisture, and competing soil microbes should be taken into consideration when conducting work with these minor pathogens.

The quantitative real-time assay based on the *tri5* gene was both specific and sensitive, and correlated well with percent infected root area in the growth-chamber assays. This assay was designed to be specific for the two primary pathogens, *F. culmorum* and *F. pseudograminearum*, in the fusarium root rot complex. It will also detect *F. graminearum*, making this technique potentially useful in fusarium head blight research. The assay did not respond to other *Fusarium* spp. or fungal saprophytes commonly isolated from wheat and barley roots in the IMW. When 37 cycles were utilized, the assay detected 61 pg with a C_t value of 35 to 36. If more cycles were used or if the annealing or extension temperature was reduced, the assay detection limit may be lowered. When utilizing 40 cycles at 60 °C in real-time PCR assays, Waalwijk et al. (2004) detected down to 0.09 pg, which is less than five genome equivalents if the predicted genome size of *F. graminearum* is 36 Mb or 0.04 pg. In a real-time assay that detected *F. graminearum* and *F. verticillioides* (Sacc.) Nirenberg, Bluhm et al. (2004) had a detection limit of 5 pg of genomic DNA with a $C_t \leq 35$. They felt that a C_t cut-off value of 35 was needed to minimize the chance of false positives.

The quantitative real-time assay based on the *tri5* gene appears to be well suited to the soilless growth-chamber assay being developed for screening germ plasm for resistance to *F. culmorum* and *F. pseudograminearum*. In addition, the real-time assay also shows potential for use with root material from field assays in the IMW. If this assay is utilized in other regions with soil-based assays, researchers will want to take into account the possibility of detecting additional *Fusarium* spp. A recent BLAST (Basic Local Alignment Search Tool) search using the sequencing of the amplified region (bases 866–928 of *Gibberella zeae*, accession No. U22464) indicated that additional *Fusarium* spp. are likely to be amplified and detectable: *F. acaciae-mearnsii* Aoki et al., *F. cortaderiae* O'Donnell et al., *F. mesoamericanum* Aoki et al., *F. austroamericanum* Aoki et al., *F. meridionale* Aoki et al., *F. asiaticum* O'Donnell et al., *F. lunulosporum* Gerlach, *F. boothii* O'Donnell et al., *F. sporotrichioides* Sherb., and *F. poae*. While these species are not common in our cereal-production system in southeastern Idaho, researchers will want to take this into consideration when applying this system in other areas.

Real-time assays recently developed in Europe (Waalwijk et al. 2004) for detecting and quantifying *Fusarium* spp. associated with fusarium head blight could potentially be utilized for root-disease research as well. However, these assays were tested only on cereal grain. To be adapted for fusarium root rot research in the IMW, these assays would need to be tested against additional *Fusarium* spp. and against saprophytes common to the IMW, along with tests on root tissue.

Despite the careful selection of healthy looking seed and surface disinfestation, we encountered some problems with *F. culmorum* seed-borne contamination in the growth-chamber assays. The seed was produced in Idaho, which typically has very little fusarium head blight. When head blight was documented by Mihuta-Grimm and Forster (1989) in Idaho, the primary causal agent was *F. culmorum*. The seed-borne contamination led to low levels of *F. culmorum* being detected in our growth-chamber assays. However, the seed-borne inoculum also led to only low levels of infection since their C_t values were always above those for experimental units inoculated with *F. culmorum*. We detected the presence of *F. culmorum* but not *F. graminearum* through the use of SCAR markers and end-point assays.

At the Arbon Valley field only 1% of the plant isolations were *F. culmorum* while at the Ririe location, 67% were *F. culmorum*. These results help confirm observations noted for these counties in a publication by Strausbaugh et al. (2004). Since *F. culmorum* was quantifiable in naturally infected plants at Ririe and undetectable at Arbon Valley, these contrasting fields helped to confirm the utility of the real-time assay. Since the field data were based on natural inoculum only, they also help to confirm that the sensitivity of the assay is sufficient for work with plants exposed to disease levels expected in commercial fields.

The real-time assay based on the *tri5* gene described in this work should allow for more sensitive root-disease research with the fusarium disease complex. The pitfalls of relying on root symptoms alone to assess pathogenicity are highlighted by the reduction in root length associated with *F. semitectum* and other *Fusarium* spp. that produce only small root lesions. This real-time assay should also prove useful in screening cereals for resistance to *F. culmorum* and *F. pseudograminearum*.

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